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SYNTHESIS, CHARACTERIZATION AND *IN-VITRO* ANTICANCER EVALUATION OF NOVEL BENZO[D]IMIDAZOLE DERIVATIVES

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Keywords:

Benzimidazole, Pyrimidine, Pyrazole, MCF-7, CaCo-2

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ABSTRACT: The novel benzimidazole derivatives were synthesized by 2-acetyl benzimidazole consent to react with various substituted aromatic aldehyde (2a–e) to obtain preferred intermediate chalcones (3a–j), these intermediates are undergo the cyclocondensation with guanidine hydrochloride and phenyl hydrazine yielded benzimidazole derivatives (4a–j and 5a–j respectively). The synthesized compounds were analyzed spectral studies to confirm their structure and *in-vitro* anticancer activity was screened in MCF-7 and CaCo-2. Among them the compounds containing benzimidazole with pyrimidine moiety (4a, 4b, 4d, 4g, 4i) and compounds containing benzimidazole with pyrazole moiety (5b, 5d, 5g, 5i) showed significant activity against MCF-7 and CaCO-2 cell line. In particular compound 4a exhibited IC_{50} 8.22µM in MCF-7 , IC50 5.67µM CaCO-2 cell line. The compound 4a induced apoptosis in Caco-2 cells as evaluated by EB/AO staining and 24 hours Caspase study revealed that compound 4a shows 2 fold activity in caspase 3 and 9 pathway, single fold activity in caspase 8 pathway, which can be regarded as promising anticancer potential.

INTRODUCTION: The benzimidazole comprises a relatively large, growing, important pharmaco-phore and privileged structure in modern drug discovery [1]. Various substituted benzimidazole derivatives have been found to possess potential anticancer properties [2]. Bis and ter - benzimidazole derivatives were extraordinarily active compounds intrusive with DNA topoisomerase I [3-4] and were also reported to their cytotoxity against the human lympholast cell line [6], breast adenocarcinoma and skin epidermoid carcinoma [7].

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3- methylthiazolo(3, 2-a)benzimidazole derivatives were also reported significant antitumor activity against the colon cancer and lower toxicity against normal fibroblast [8].

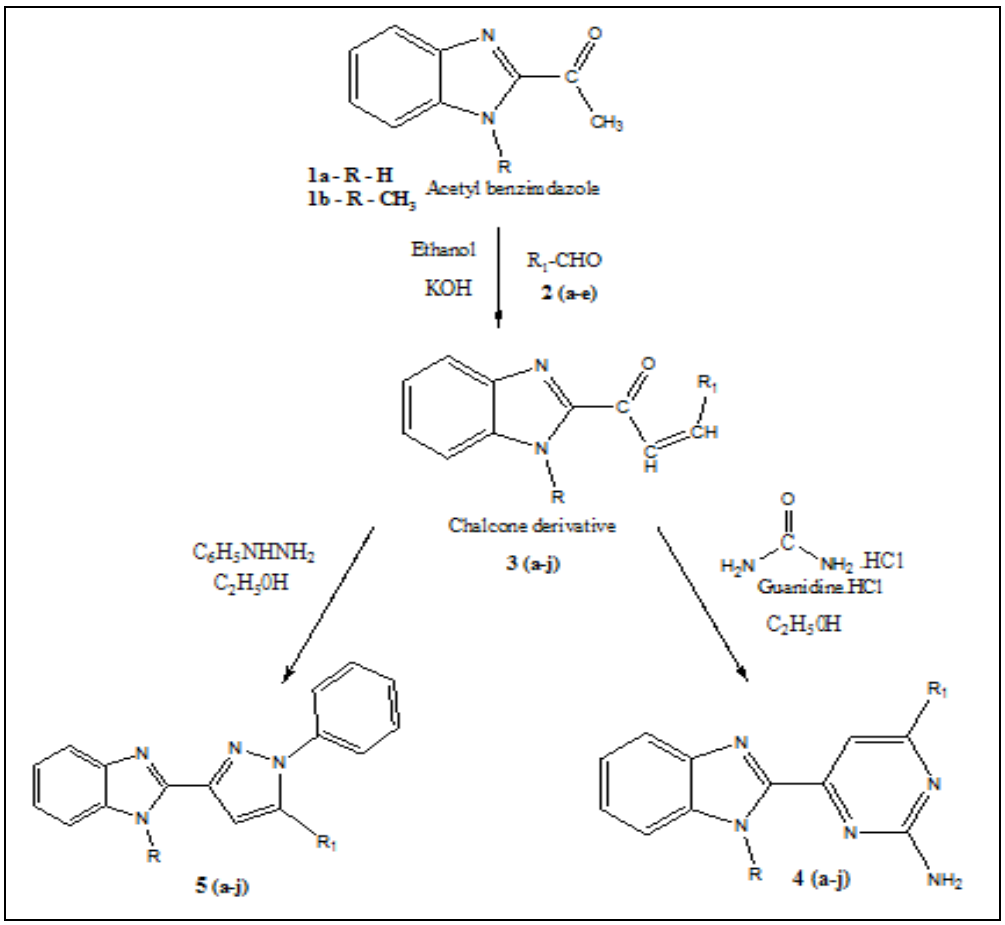
A rational approach for design the next generation inhibitors, benzimidazole derivatives, for example, Albendazole, a benzimidazole derivative clinically used as an anthemintic agent, can also prove in both *in vitro* and *in vivo* experimental condition inhibit the hepatocellular carcinoma proliferation [9]. Cyclic amine containing benzimidazole carbox-amide 2-(1-propylpiperidin-4-yl)-1H- benzimida-zole-4- carboxamide [10] were vastly more potent as poly(ADP-ribose) polymerase inhibitor and also (4-hydroxyphenyl)benzimidazole-4-carboxamide 2-(4-oxadiazolyphenyl) derivatives were showed that enhance effect in chemotherapy and radiation

therapy *in-vitro* [11] and *in-vivo* [11]. Tricyclic benzimidazole derivative [AG14361], which is high – potency PARP-1 inhibitor, has been developed and used *in-vivo* at non-toxic dose to enhance the effect of chemotherapy and radiation therapy in human cancer cell cultures and xenografts [13, 14].

Pyrimidine is a unique molecule possesses wide range of biological activity and anticancer property like 5-flourouracil [15, 16] and pyrazole nucleus are also potent medicinal scaffolds and exhibit a full spectrum of biological and anticancer activities [17, 18]. Both are known to be associated with multiple anticancer properties and biological activity. From the structural and chemistry point of view, literature survey revealed that the utility of pyrimidine and pyrazole derivatives are considered as useful synthon with benzimidazole derivatives could have anticancer potential scaffold. Based on all these findings and literature review, the aim of present work was focused to synthesis the benzimidazole-pyrimidine conjugate and benzimidazole-pyrazole conjugate with backbone scaffold of chalcones, in order to investigate there *in vitro* anticancer activities.

MATERIALS AND METHODS:

Synthesis Scheme:



Synthesis of Benzimidazolyl Chalcones (3a-e): Weighed 0.01 molar of 2-acteyl benzimidazole (1a) and 10m 30 % potassium hydroxide solution was taken in a round bottom flask to this add 0.012 molar of various aromatic aldehyde (2a-e) namely, benzadehyde (2a), pyridine aldehyde (2b), 3-chloro-4-methoxy benzadehyde (2c), 3,4-dimethoxy benzadehyde (2d), anthracene aldehyde (2e) separately, with stirring then reflux the mixture for 2 hours, cool to room temperature and pour in to a beaker containing ice cold water, with stirring to get a product. The precipitated chacolnes was filtered with ice cold water, washed dried and recrystalized from ethanol. The chemical names of above synthesized benzimidazoly chalcones as follows: (3a-e).

- 1-(1H-benzo[d]imidazole-2-yl)- 3-phenylprop-2-en-1-one
- 1-(1H- benzo[d]imidazole- 2-yl)-3-(pyridine- 4-yl)prop-2-en-1-one
- 1-(1H- benzo[d]imidazole-2-yl)-3-(3-chloro-4-methoxyphenyl)prop-2-en-1-one
- 1-(1H-benzo[d]imidazole-2-yl)-3-(3,4-dimetho xyphenyl)prop-2-en-1-one
- 3-(anthracen-10-yl)1-(1H-benzo[d]imidazole-2-yl)prop-2-en-1-one

Synthesis of 1-methyl-benzimidazolyl Chalcones (3f-j): Weighed 0.01 molar of 1-methyl-2-acteyl benzimidazole (1b) and 10m 30% potassium hydroxide solution was taken in a round bottom fask to this add 0.012 molar of various aromatic aldehyde (2a-e) namely, benzadehyde (2a), pyridine aldehyde (2b), 3-chloro-4-methoxy benzadehyde (2c), 3,4-dimethoxy benzadehyde (2d), anthracene aldehyde (2e) separately, with stirring then reflux the mixture for 2 hours, cool to room temperature and pour in to a beaker containing ice cold water, with stirring to get a product. The precipitated chacolnes was filtered with ice cold water, washed dried and recrystalized from ethanol. The chemical names of above synthesized benzimidazoly chalcones as follows: (3f-j).

- 1-(1-methyl-1H-benzo[d]imidazole-2-yl)-3-phe nylprop-2-en-1-one
- 1-(1-methyl-1H-benzo[d]imidazole-2-yl)-3-(py ridine-4-yl)prop-2-en-1-one
- 3-(3-chloro-4-methoxyphenyl) 1-(1-methyl-1H-benzo[d]imidazole-2-yl)-prop-2-en-1-one
- 3-(3,4-dimethoxyphenyl)-1-(1-methyl-1H-ben zo[d]imidazole-2-yl)-prop-2-en-1-one
- 3-(anthracen-10-yl)1-(1-methyl-1H-benzo[d]im idazole-2-yl)prop-2-en-1-one

Synthesis of Benzo[d]imidazol-2-yl Pyrimidine Derivatives (4a-e): A mixture of compounds 3(a-e) (0.001mol) was condensed with guninidine hydrochloride (0.001 mol) in presences of potassium hydroxide (0.002 mol) in absolute ethanol (5ml) at reflux on a water bath for 3 hours. The reaction mixture was cooled at room temperature and poured in ice cold water. Solid product was filtered and washed with water, dried and recrystallized to give 4(a-e), respectively.

Synthesis of 1-methyl-benzo[d]imidazol-2-yl pyrimidine Derivatives (4f-j): A mixture of compounds 3(f-j) (0.001mol) was condensed with guninidine hydrochloride (0.001 mol) in presences of potassium hydroxide (0.002 mol) in absolute ethanol (5ml) at reflux on a water bath for 3 hours. The reaction mixture was cooled at room temperature and poured in ice cold water. Solid product was filtered and washed with water, dried and recrystallized to give 4(f-j**),** respectively.

Synthesis of (pyrazol-3-yl)-benzo[d]imidazole Derivatives (5a-e): A mixture of compounds 3(a-e) (0.001mol) was condensed with phenyl hydrazine (0.001 mol) in presences of potassium hydroxide (0.002 mol) in absolute ethanol (5ml) at reflux on a water bath for 3 hours. The reaction mixture was cooled at room temperature and poured in ice cold water. Solid product was filtered and washed with water, dried and recrystallized to give 5(a-e), respectively.

Synthesis of (pyrazol-3-yl)-1-methyl-1H-benzo [d]imidazole Derivatives (5f-j): A mixture of compounds 3(f-j) (0.001 mol) was condensed with phenyl hydrazine (0.001 mol) in presences of potassium hydroxide (0.002 mol) in absolute ethanol (5ml) at reflux on a water bath for 3 hours. The reaction mixture was cooled at room temperature and poured in ice cold water. Solid product was filtered and washed with water, dried and recrystallized to give 5(f-j), respectively.

***In-vitro* Cytotoxic Evaluation against MCF- and Caco-2 Cell Line:**

Cell Culture: MCF-7 (Human breast cancer cell line) and Caco-2 (Human colon cancer cell line) were obtained from NCCS Pune. It was maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum (FBS) [19, 20]. Approximately 2000 cells/well were seeded in 96 well pate using culture medium, the viability was confirmed. After 24 hrs, the fresh medium with the test or standard compound were added at respective wells and kept incubation for 72 hrs. After incubation the following assays were performed. After 72 hrs of the drug treatment the fresh medium was changed again for all groups and 10µl of MTT (5mg/ml stock solution) was added and the plates were incubated for an additional 4 hrs. The medium was discarded and the formazan

blue, which was formed in the cells, was dissolved with 100µ of DMSO. The optical density was measured at 570nm.

The percentage toxicity was calculated by using following formula. Graph pad prism software was used to calculate IC_{50} of the extracts.

% Toxicity = 1- Treated cells / Untreated cells X 100

RESULT AND DISCUSSION: Melting points of the synthesized compounds were determined using melting point apparatus and were found uncorrected. The progress of the reaction was monitored by thin layer chromatography using pre-coated sheet silica gel MERCK and was visualized by UV lamp. The IR spectra of synthesized compounds were recorded using KBr pellets in the range 4000-400cm^{-1} on the Fourier Transform IR Spectrophotometer (Model - Shimadzu) and frequencies were recorded in wave numbers (cm^{-1}). The ^{1}H NMR spectra were recorded by BRUKER AV III 500MHz (Indian Institute of Technology, Madras). Chemical shifts (δ) are reported in parts per million (ppm). Down fied from internal reference tetramethylsilane (TMS). Mass spectrum was recorded by LC-MS model - JEOL GC MATE (Indian Institute of Technology, Madras).

Spectral Discussion:

4-(1H-benzo[d]imidazol-2-yl)-6-phenylpyrimidin-2-amine (4a): R_f = 0.75, MP = 146 °C, IR (KBr) cm^{-1}: 3378 cm^{-1} (NH_2 Stretching), 3523 cm^{-1} (NH Stretching), 3201 cm^{-1} (Aromatic CH Stretching), 1453 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 988 cm^{-1} (Aromatic C-H Stretching), 1303 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm)**:** 7.48 - 8.20 (m, 4H, Benzimidazole), 7.21 - 7.47 (m, 5H, Ar-H), 4.17 (s, 2H, Amino pyridine), 5.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d6) δ (ppm)**:** δ 154.79 (Benzimidazole-C), 124.02 (Benzimidazole - CH), 126.87 (Benzene - CH), 133.02 (Benzene - C), 163.79 (Pyrimidine - C), 95.45 (Pyrimidine - CH). Mass: m/z: 287.32.

4- (1H-benzo[d]imidazol- 2- yl)- 6- (pyridin-4-yl) pyrimidin-2-amine (4b): R_f = 0.65, MP = 156 °C, IR (KBr) cm^{-1}**:** 3412 cm^{-1} (NH_2 Stretching), 3556 cm^{-1} (NH Stretching), 3140 cm^{-1} (Aromatic CH Stretching), 1439 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 915 cm^{-1} (Aromatic C-H Stretching), 1290 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.71 – 8.16 (m, 4H, Benzimidazole), 7.08 - 7.11 (m, 5H, Ar-H), 4.27 (s, 2H, Amino pyridine), 5.17 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d6) δ (ppm): δ 154.95 (Benzimidazole-C), 121.81 (Benzimidazole - CH), 126.87, 163.79 (Pyrimidine - C), 95.45 (Pyrimidine - CH), 146.04 (Pyridine-C), 121.81(Pyridine-CH). Mass: m/z: 288.31.

4-(1H-benzo[d]imidazol- 2-yl)- 6- (3- chloro-4-methoxyphenyl)pyrimidin-2-amine (4c): R_f = 0.40, MP = 216 °C, IR (KBr) cm^{-1}: 3378 cm^{-1} (NH_2 Stretching) , 3523 cm^{-1} (NH Stretching), 3201 cm^{-1} (Aromatic CH stretching), 1418 cm^{-1} (Aromatic C = C Stretching), 1589 cm^{-1} (Aromatic C = N Stretching), 875 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.48 - 8.26 (m, 4H, Benzimidazole), 6.94 - 7.40 (m, 4H, Ar-H), 4.09 (s, 2H, Amino pyridine), 5.13 (s, 1H,), 3.46- 3.62 (m, 6H, 2 - OCH_3). ^{13}C-NMR (400 MHz, DMSO-d6) δ in ppm: δ 154.04 (Benzimidazole - C), 124.02 (Benzimidazole - CH), 126.87 (Benzene - CH), 133.02 (Benzene - C), 163.95 (Pyrimidine - C), 93.45 (Pyrimidine - CH) Mass: m/z: 351.79

4-(1H-benzo[d]imidazol-2-yl)- 6- (3,4- dimethoxy phenyl)pyrimidin-2-amine(4d): R_f = 0.80, MP = 140 °C, IR (KBr) cm^{-1}: 3484 cm^{-1} (NH_2 Stretching), 3398 cm^{-1} (NH Stretching), 3284 cm^{-1} (Aromatic CH stretching), 1585 cm^{-1} (Aromatic C=C Stretching) , 1589 cm^{-1} (Aromatic C=N Stretching), 879 cm^{-1} (Aromatic C-H Bending), 1383 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.21 - 7.47 (m, 4H, Benzimidazole), 7.48 - 8.20 (m, 5H, Ar-H), 4.17 (s, 2H, Amino pyridine), 5.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.81 (Benzimidazole - C), 115.27 (Benzimidazole - CH), 163.81 (Pyrimidine - C), 95.62 (Pyrimidine - CH) Mass: m/z: 347.37.

4-(anthracen-10-yl)- 6- (1H-benzo[d]imidazol- 2-yl)pyrimidin-2-amine (4e): R_f = 0.70, MP = 190 °C, IR (KBr) cm^{-1}: 3313 cm^{-1} (NH_2 Stretching), 3370 cm^{-1} (NH Stretching), 3100 cm^{-1} (Aromatic CH stretching), 1428 cm^{-1} (Aromatic C=C Stretching), 1534 cm^{-1} (Aromatic C=N Stretching), 860 cm^{-1} (Aromatic C-H Bending), 1370 cm^{-1}

(Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.46 - 7.87 (m, 4H, Benzimidazole), 7.25 - 7.49 (m, 10H, Ar-H), 4.17 (s, 2H, Amino pyridine), 5.10 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d6) δ in ppm: δ 154.44 (Benzimidazole - C), 123.02 (Benzimida zole - CH), 163.32 (Pyrimidine - C), 95.70 (Pyrimidine - CH). Mass: m/z: 387.15

4-(1-methyl-1H-benzo[d]imidazol-2-yl)-6-phenyl pyrimidin-2-amine (4f): R_f = 0.75, MP = 152 °C, IR (KBr) cm^{-1}: 3378 cm^{-1} (NH_2 Stretching), 3523 cm^{-1} (NH Stretching), 3201 cm^{-1} (Aromatic CH stretching), 1453 cm^{-1} (Aromatic C= C Stretching), 1589 cm^{-1} (Aromatic C= N Stretching), 988 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.48 - 8.21 (m, 4H, Benzimidazole), 7.19 - 7.47 (m, 5H, Ar-H), 4.17 (s, 2H, Amino pyridine), 4.90 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.79 (Benzimidazole - C), 123.02 (Benzimidazole - CH), 119.87(Benzene - CH), 133.02 (Benzene - C), 163.79 (Pyrimidine -C), 95.17 (Pyrimidine - CH), 32.04 (Aliphatic - CH_3) 78.89 (Aliphatic - CH) Mass: m/z: 301.13.

4-(1-methyl-1H-benzo[d]imidazol-2-yl)-6-(pyrid ine-4-yl)pyrimidin-2-amine (4g): R_f = 0.65, MP = 154 °C, IR (KBr) cm^{-1}: 3410 cm^{-1} (NH_2 Stretching), 3556 cm^{-1} (NH Stretching), 3140 cm^{-1} (Aromatic CH stretching), 1439 cm^{-1} (Aromatic C=C Stretching) , 1589 cm^{-1} (Aromatic C=N Stretching), 915 cm^{-1} (Aromatic C-H Bending), 1290 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.66 - 7.71 (m, 4H, Benzimidazole), 7.08 - 7.11 (m, 5H, Ar-H), 4.27 (s, 2H, Amino pyridine), 5.17 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.90 (Benzimidazole - C), 123.71 (Benzimidazole - CH), 163.36 (Pyrimidine - C), 95.17 (Pyrimidine - CH) 31.04 (Aliphatic - CH_3). Mass: m/z: 302.13.

4-(3-chloro-4-methoxyphenyl)- 6-(1- methyl- 1H-benzo[d]imidazol- 2-yl)pyrimidin- 2- amine (4h): R_f = 0.82, MP = 204 °C, IR (KBr) cm^{-1}: 3484 cm^{-1} (NH_2 Stretching), 3398 cm^{-1} (NH Stretching), 3284 cm^{-1} (Aromatic CH stretching), 1585 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 879 cm^{-1} (Aromatic C-H Bending), 1383 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.47 - 8.20 (m, 4H, Benzimidazole), 7.48 - 8.26 (m, 5H, Ar-H), 4.76 (s, 2H, Amino pyridine), 5.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.04 (Benzimidazole - C), 124.02 (Benzimidazole - CH), 126.87 (Benzene -NCH), 133.02 (Benzene - C), 163.95 (Pyrimidine - C), 93.45 (Pyrimidine - CH), 55.71 (Aliphatic - CH_3). 31.89 (Aliphatic - CH_3). Mass: m/z: 365.15.

4-(3,4-dimethoxyphenyl)-6- (1-methyl-1H- benzo [d]imidazol-2-yl)pyrimidin-2-amine (4i): R_f = 0.68, MP = 202 °C, IR (KBr) cm^{-1}: 3378 cm^{-1} (NH_2 Stretching), 3523 cm^{-1} (NH Stretching), 3201 cm^{-1} (Aromatic CH stretching), 1418 cm^{-1} (Aromatic C= C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 875 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.65 - 8.19 (m, 4H, Benzimidazole), 7.39 - 7.54 (m, 4H, Ar-H), 4.46 (s, 2H, Amino pyridine), 5.10 (s, 1H), 3.63- 3.72 (m, 6H, 2 - OCH_3). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.81 (Benzimidazole - C), 115.27 (Benzimidazole - CH), 163.81 (Pyrimidine - C), 95.62 (Pyrimidine - CH), 56.71 (Aliphatic - CH_3). 31.89 (Aliphatic - CH_3). Mass: m/z: 361.15

4-(anthracen-10-yl)- 6- (1- methyl- 1H- benzo[d] imidazol-2-yl)pyrimidin-2-amine (4j): R_f = 0.60, MP = 208 °C, IR (KBr) cm^{-1}: 3313 cm^{-1} (NH_2 Stretching), 3375 cm^{-1} (NH Stretching), 3099 cm^{-1} (Aromatic CH stretching), 1428 cm^{-1} (Aromatic C= C Stretching), 1534 cm^{-1} (Aromatic C=N Stretching), 860 cm^{-1} (Aromatic C-H Bending), 1370 cm^{-1} (Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.46 - 7.87 (m, 4H, Benzimidazole), 7.25 - 7.49 (m, 10H, Ar-H), 4.17 (s, 2H, Amino pyridine), 5.10 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.44 (Benzimidazole - C), 123.02 (Benzi-midazole - CH), 163.32 (Pyrimidine - C), 95.70 (Pyrimidine - CH), 31.90 (Aliphatic - CH_3). Mass: m/z: 401.46.

2-(1,5- diphenyl- 1H-pyrazol-3-yl)- 1H-benzo[d] imidazole (5a): R_f = 0.7, MP = 168 °C, IR (KBr) cm^{-1}: 3520 cm^{-1} (NH Stretching), 3100 cm^{-1} (Aromatic CH stretching), 1505 cm^{-1} (Aromatic C=C Stretching), 1534 cm^{-1} (Aromatic C=N Stretching), 880 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching), 1168 cm^{-1}

(C-O-C Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.75 - 7.77 (m, 4H, Benzimidazole), 6.96 - 7.51 (m, 10H, Ar-H), 5.22 (s, 1H,), 4.02- 4.06 (m, 6H, 2 - OCH_3). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 138.37 (Benzimidazole - C), 123.01 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 163.79 (Pyrimidine - C), 95.45 (Pyrimidine - CH). Mass: m/z: 336.14.

2-(1-phenyl-5-(pyridin-4-yl)- 1H-pyrazol- 3-yl)-1 H-benzo[d]imidazole (5b): R_f = 0.62, MP = 176 °C, IR (KBr) cm^{-1}: 3523 cm^{-1} (NH Stretching), 3094 cm^{-1} (Aromatic CH stretching), 1505 cm^{-1} (Aromatic C= C Stretching), 1534 cm^{-1} (Aromatic C=N Stretching), 880 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching), 1168 cm^{-1} (C-O-C Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.75 - 7.77 (m, 4H, Benzimidazole), 6.96 - 7.51 (m, 10H, Ar-H), 5.22 (s, 1H,), 4.02- 4.06 (m, 6H, 2 - OCH_3). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 138.37 (Benzimidazole - C), 123.01 (Benzimidazole - CH), 126.38 (Pyrazole - C), 108.98 (Pyrazole - CH), 126.87(Benzene - CH), 139.02 (Benzene - C), 149.79 (Pyridine - C), 121.45 (Pyridine - CH). Mass: m/z: 337.13.

2-(5-(3-chloro-4-methoxyphenyl)- 1- phenyl- 1H-pyrazol-3-yl)-1H-benzo[d]imidazole (5c): R_f = 0.55, MP = 192 °C, IR (KBr) cm^{-1}: 3484 cm^{-1} (NH Stretching), 3100 cm^{-1} (NH Stretching), 3284 cm^{-1} (Aromatic CH stretching), 1585 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 879 cm^{-1} (Aromatic C-H Bending), 1383 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.21 - 7.47 (m, 4H, Benzimidazole), 7.48 - 8.20 (m, 5H, Ar-H), 5.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.01 (Benzimidazole - C), 123.37 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 55.40 (Aliphatic - CH_3). Mass: m/z: 400.11.

2-(5-(3,4-dimethoxyphenyl)- 1- phenyl- 1H-pyrazol-3-yl)-1H-benzo[d]imidazole (5d): R_f = 0.45, MP = 182 °C, IR (KBr) cm^{-1}: 3391 cm^{-1} (NH Stretching), 3099 cm^{-1} (Aromatic CH stretching), 1453 cm^{-1} (Aromatic C=C Stretching), 1623 cm^{-1} (Aromatic C=N Stretching), 759 cm^{-1} (Aromatic C-H Bending), 1505 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.11 - 7.33 (m, 4H, Benzimidazole), 7.34 - 7.42 (m, 13H, Ar-H), 5.11 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.01 (Benzimidazole - C), 123.37 (Benzimidazole - CH), 142.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 56.24 (Aliphatic - CH_3). Mass: m/z: 396.16

2-(5-(anthracen-10-yl)- 1- phenyl-1H-pyrazol- 3-yl)-1H-benzo[d]imidazole (5e): R_f = 0.70, MP = 162 °C, IR (KBr) cm^{-1}: 3370 cm^{-1} (NH Stretching), 3100 cm^{-1} (Aromatic CH stretching), 1428 cm^{-1} (Aromatic C=C Stretching), 1534 cm^{-1} (Aromatic C=N Stretching), 860 cm^{-1} (Aromatic C-H Bending), 1370 cm^{-1} (Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.46 - 7.87 (m, 4H, Benzimidazole), 7.25 - 7.49 (m, 10H, Ar-H), 5.10 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 138.37 (Benzimidazole - C), 123.01 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C). Mass: m/z: 436.17.

2-(1,5-diphenyl-1H-pyrazol-3-yl)- 1- methyl- 1H-benzo[d]imidazole (5f): R_f = 0.6, MP = 164 °C, IR (KBr) cm^{-1}: 3523 cm^{-1} (NH Stretching), 3195 cm^{-1} (Aromatic CH stretching), 1399 cm^{-1} (Aromatic C=C Stretching), 1625 cm^{-1} (Aromatic C=N Stretching), 888 cm^{-1} (Aromatic C-H Bending), 1324 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.26 - 7.70 (m, 4H, Benzimidazole), 7.72 - 8.48 (m, 4H, Ar-H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 141.45 (Benzimidazole - C), 115.30 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87 (Benzene - CH), 133.02 (Benzene - C), 31.24 (Aliphatic - CH_3). Mass: m/z: 350.15.

1-methyl- 2- (1-phenyl-5- (pyridin-4-yl)- 1H-pyra zol-3-yl)-1H-benzo[d]imidazole (5g): R_f = 0.67, MP = 210 °C, IR (KBr) cm^{-1}: 3556 cm^{-1} (NH Stretching), 3140 cm^{-1} (Aromatic CH stretching), 1439 cm^{-1} (Aromatic C= C Stretching, 1589 cm^{-1} (Aromatic C= N Stretching), 915 cm^{-1} (Aromatic C-H Bending), 1290 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.66 - 7.71 (m, 4H , Benzimidazole), 7.08 - 7.11 (m, 5H , Ar-H), 5.17 (s, 1H). ^{13}C-NMR **(**400 MHz,

DMSO-d_6) δ in ppm: δ 138.37 (Benzimidazole - C), 123.01 (Benzimidazole - CH), 126.38 (Pyrazole - C), 108.98 (Pyrazole - CH), 126.87(Benzene - CH), 139.02 (Benzene - C), 141.79 (Pyridine -C), 121.45 (Pyridine - CH), 31.24 (Aliphatic - CH_3). Mass: m/z: 351.15.

2-(5-(3-chloro-4-methoxyphenyl)- 1 -phenyl- 1H-pyrazol-3-yl)- 1- methyl- 1H- benzo[d] imidazole (5h): R_f = 0.75, MP = 169 °C, IR (KBr) cm^{-1}: 3484 cm^{-1} (NH_2 Stretching), 3398 cm^{-1} (NH Stretching), 3284 cm^{-1} (Aromatic CH stretching), 1585 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 879 cm^{-1} (Aromatic C-H Bending), 1383 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.47- 8.20 (m, 4H, Benzimidazole), 7.48 - 8.26 (m, 5H, Ar-H), 4.76 (s, 2H, Amino pyridine), 5.13 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 138.01 (Benzimidazole - C), 123.37 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 31.24 (Aliphatic - CH_3), 55.24(Aliphatic - CH_3). Mass: m/z: 414.12.

2-(5-(3,4-dimethoxyphenyl)-1-phenyl-1H-pyrazol-3-yl)-1-methyl-1H-benzo[d]imidazole (5i): R_f = 0.62, MP = 203 °C, IR (KBr) cm^{-1}: 3378 cm^{-1} (NH_2 Stretching), 3523 cm^{-1} (NH Stretching), 3201 cm^{-1} (Aromatic CH stretching), 1418 cm^{-1} (Aromatic C=C Stretching), 1589 cm^{-1} (Aromatic C=N Stretching), 875 cm^{-1} (Aromatic C-H Bending), 1303 cm^{-1} (Aromatic C-N Stretching), 1118 cm^{-1} (C-Cl Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.65 - 8.19 (m, 4H, Benzimidazole), 7.39 - 7.54 (m, 4H, Ar-H), 5.10 (s, 1H), 3.63- 3.72 (m, 6H, 2 - OCH_3). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 154.01 (Benzimidazole - C), 123.37 (Benzimidazole - CH), 142.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 31.24(Aliphatic - CH_3), 56.24 (Aliphatic - CH_3). Mass: m/z: 410.17.

2-(5-(anthracen-10-yl)-1- phenyl- 1H-pyrazol- 3-yl)-1-methyl-1H-benzo[d]imidazole (5j): R_f = 0.4 MP = 205 °C, IR (KBr) cm^{-1}: 3362 cm^{-1} (NH Stretching), 3179 cm^{-1} (Aromatic CH stretching), 1590 cm^{-1} (Aromatic C=C Stretching), 1625 cm^{-1} (Aromatic C=N Stretching), 873 cm^{-1} (Aromatic C-H Bending), 1246 cm^{-1} (Aromatic C-N Stretching). ^{1}H NMR (500 MHz, DMSO) δ (ppm): 7.20 - 7.59 (m, 4H, Benzimidazole), 7.60 - 8.20 (m, 4H, pyridine), 6.51 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d_6) δ in ppm: δ 138.37 (Benzimidazole - C), 123.01 (Benzimidazole - CH), 143.38 (Pyrazole - C), 107.98 (Pyrazole - CH), 126.87(Benzene - CH), 133.02 (Benzene - C), 31.24 (Aliphatic - CH_3). Mass: m/z: 450.18.

***In-vitro* Anticancer Activity:** *In-vitro* anticancer activity was investigated for all synthesised compounds to MCF-7 and CaCo-2 in the different doses and found the concentration required for the 50% cell death (IC_{50}). The ability of the test compounds to inhibit cell growth was listed in **Table 3**. The general structure-activity relationship of the benzimidazole analogues can be summarized as below: Comparison of the cytotoxicity of benzo [d]imidazolyl pyrimidine derivatives and benzo [d]imidazolyl Pyrazole derivatives indicates that the importances of substituted benzimidazole with pyrimidine derivatives (4a-j) generally enhance the cytotoxicity against MCF-7 and CaCo-2 cancer cells. Addition of pyrazole moiety instead of pyrimidine moiety with benzimidazole nucleus to give compound **(**5a-j) was clearly detrimental to the cytotoxicity in both MCF-7 and CaCo-2 cancer cells. It should be noted that compound (5a-j) has a lowest activity than compound (4a-j).

For compound 4(a-e) (2-position of benzimidazole core was substituted by pyrimidine with various aromatic moiety), it indicated that introducing the various substituent group to aromatic ring of pyrimidine moiety would not affect their efficacy for MCF-7 and CaCo-2 cell lines, but compound 4a, 4b, 4c, 4d would affect their efficacy too much for CaCo-2 cell line: 4a (pyrimidine ring was substituted with benzene) (IC_{50} = 5.67μM) was 5.29-fold much stronger than 4e and 4f (IC_{50} => 30μM), 4b (pyrimidine ring was substituted with pyridine) (IC_{50} = 9.56μM) was 3.13-fold stronger than 4e and 4f (IC_{50} => 30μM), 4d (pyrimidine ring was substituted with two 3,4-dimethoxy benzene group) (IC_{50} = 12.33μM) was 2.43-fold much stronger than 4e and 4f (IC_{50} => 30μM), 4c (pyrimidine ring was substituted with two 3-chloro4-methoxy benzene group) (IC_{50} = 28.40μM) was 1.05-fold much stronger than 4e and 4f (IC_{50} => 30μM), 4a was also 3.64-fold much stronger than 4e and 4f (IC_{50} => 30μM) against MCF-7.

For compounds 4(f-j) (2-position of 1-methyl-benzimidazole core was substituted by pyrimidine with various aromatic moiety), it indicated that introducing the various substituent group to aromatic ring of pyrimidine moiety not much influence for MCF-7 and CaCo-2 cell lines, but compound 4g(IC_{50} = 16.23μM), 4h(IC_{50} = 25.50μM), 4j(IC_{50} = 21.89μM) would affect their efficacy too much in CaCo-2 cell line compare with MCF-7 cell line.

For compound 5(a-e) (2-position of benzimidazole core was substituted by pyrazole with various aromatic moiety), it indicated that introducing the various substituent group to aromatic ring of pyrazole moiety which affect their efficacy for MCF-7 and CaCo-2 cell lines. 5b (IC_{50} = 9.79μM), 5d (IC_{50} = 12.33μM) would affect their efficacy too much in CaCo-2 cell line compare with MCF-7 cell line.

For compound 5(f-j) (2-position of 1-methyl-benzimidazole core was substituted by pyrimidine with various aromatic moiety), it indicated that introducing the various substituent group to aromatic ring of pyrazole moiety not much influence for MCF-7 and CaCo-2 cell lines. 5g (IC_{50} = 17.32μM), 5i (IC_{50} = 18.35μM) would affect their efficacy too much in CaCo-2 cell line compare with MCF-7 cell line.

TABLE 1: IC_{50} OF THE TESTED COMPOUNDS AGAINST OF MCF-7 AND CACO-2 CELL LINE - BENZO [D] IMIDAZOLE PYRIMIDINE DERIVATIVES

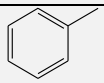
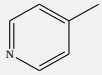
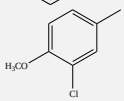
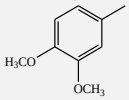
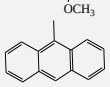
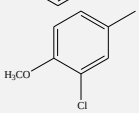
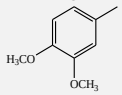
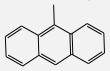
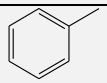
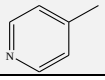
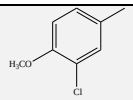
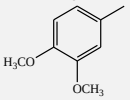
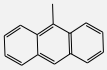
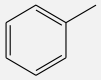
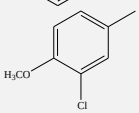
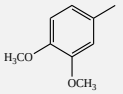
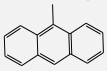
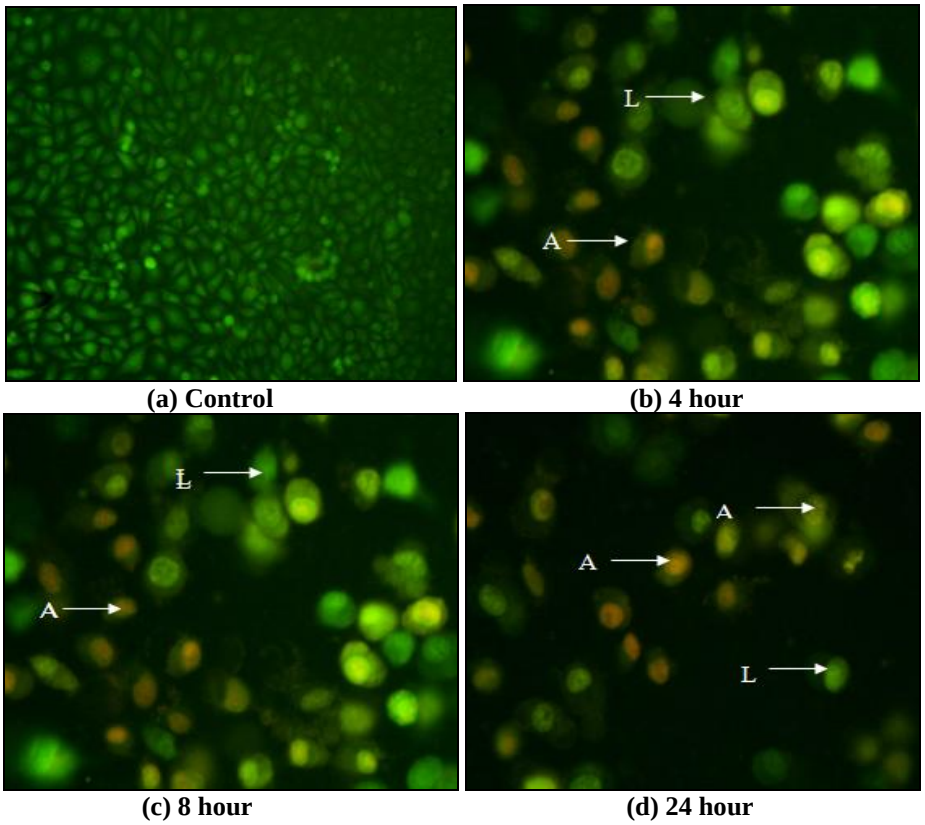
S. No	Compound code	Substituent R	Substituent R_1	Molecular Formula	IC_{50} ± SD (μM) MCF-7	CaCo-2
1	4a	H		$C_{17}H_{13}N_5$	8.22 ± 1.48	5.67 ± 1.25
2	4b	H		$C_{16}H_{12}N_6$	10.43 ± 1.45	9.56 ±1.33
3	4c	H		$C_{19}H_{17}N_5O_2$	>30	28.40 ± 248
4	4d	H		$C_{18}H_{14}ClN_5O$	13.05 ±2.07	12.33 ± 1.80
5	4e	H		$C_{25}H_{17}N_5$	>30 ± 2.87	>30 ± 2.98
6	4f	CH_3		$C_{18}H_{15}N_5$	>30 ± 2.66	>30 ± 2.43
7	4g	CH_3		$C_{17}H_{14}N_6$	18.56 ± 2.82	16.23 ± 1.24
8	4h	CH_3		$C_{19}H_{16}ClN_5O$	>30 ± 2.19	25.50 ± 2.74
9	4i	CH_3		$C_{20}H_{19}N_5O_2$	25.11 ± 2.44	21.89 ± 2.35
10	4j	CH_3		$C_{26}H_{19}N_5$	>30 ± 2.80	>30 ± 2.06
		5-Fluorouracil			7.26 ± 2.30	5.23 ± 2.36

TABLE 2: IC_{50} OF THE TESTED COMPOUNDS AGAINST OF MCF-7 AND CACO-2 CELL LINE - BENZO [D] IMIDAZOLE PYRAZOLE DERIVATIVES

S. No	Compound code	Substituent R	Substituent R_1	Molecular Formula	IC_{50} ± SD (μM) MCF-7	CaCo-2
11	5a	H		$C_{22}H_{16}N_4$	26.65 ± 2.32	28.45 ± 2.59
12	5b	H		$C_{21}H_{15}N_5$	12.79 ± 2.20	9.788 ± 1.48

13	5c	H		$C_{23}H_{17}ClN_4O$	>30 ± 2.86	>30 ± 2.48
14	5d	H		$C_{24}H_{20}N_4O_2$	15.34 ± 2.67	13.27 ± 1.56
15	5e	H		$C_{30}H_{20}N_4$	>30 ± 2.52	>30 ± 2.33
16	5f	CH_3		$C_{23}H_{18}N_4$	>30 ± 2.41	>30 ± 2.69
17	5g	CH_3		$C_{22}H_{17}N_5$	19.04 ± 2.56	17.32 ± 2.27
18	5h	CH_3		$C_{24}H_{19}ClN_4O$	>30 ± 2.38	29.76 ± 2.64
19	5i	CH_3		$C_{25}H_{22}N_4O_2$	21.73 ± 2.46	18.35 ± 2.54
20	5j	CH_3		$C_{31}H_{22}N_4$	>30 ± 2.58	>30 ± 2.62
		5-Fluorouracil			7.26 ± 2.30	5.23 ± 2.36

Apoptosis Studies: Apoptosis studies were performed with a staining method utilizing acridine orange (AO) and ethidium bromide (EB). CaCo-2 cells were incubated in the absence or presence of the test compound 4a and the indicated synthesized compounds at their respective IC_{50} concentration at 37 °C and 5% CO_2 for 48 hr. After 48 hr, cells were stained with AO/EB solution (100 µg/mL AO, 100µg/mL EB). Then the cells were observed under a fluorescence microscope.



(a) Control **(b) 4 hour**

(c) 8 hour **(d) 24 hour**

FIG. 1: EB/AO METHOD FOR CACO-2 CELLS

White arrows indicate "L" = live cells; "A" = apoptotic cells; CaCo-2 cells were treated with 5.67µM compound 4a for induction of apoptosis. The compound 4a induced apoptosis in Caco-2 cells as evaluated by EB/AO staining.

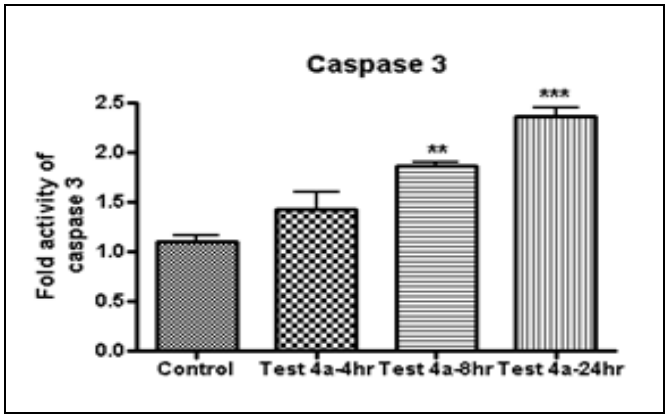


FIG. 2: FOLD ACTIVITY OF CASPASE 3

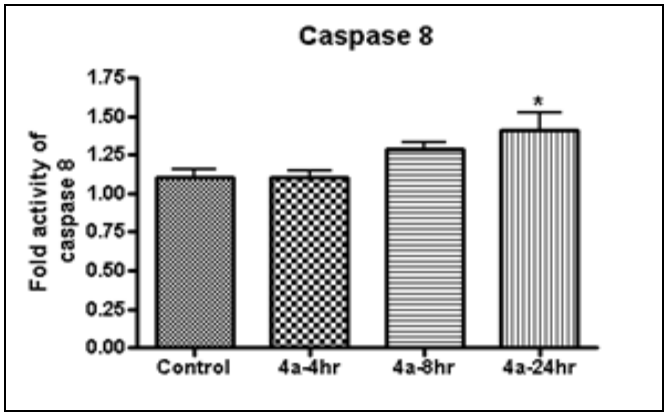


FIG. 3: FOLD ACTIVITY OF CASPASE 8

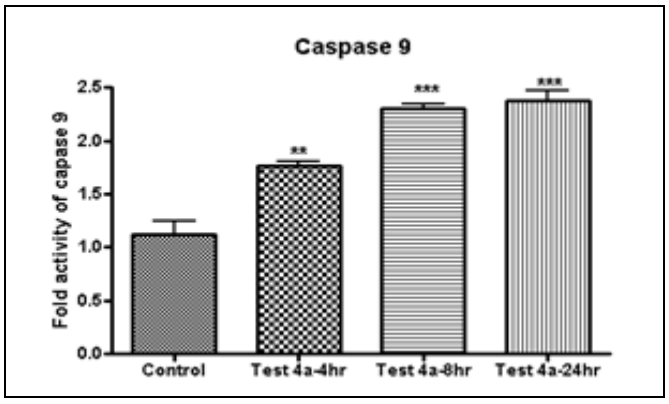


FIG. 3: FOLD ACTIVITY OF CASPASE 9

24 hours Caspase study revealed that compound 4a shows 2 fold activities in caspase 3 and 9 pathway, single fold activity in caspase 8 pathways.

CONCLUSION: To conclude, various substituted novel benzimidazole derivatives were synthesised by using incorporated with different heterocycles like pyrimidine, pyrazole and backbone of chalcones. The synthesised compounds were screened for their *in vitro* anticancer activities results revealed that the presence substituted benzimidazole derivative could have the anticancer potential of the scaffold.

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CONFLICTS OF INTEREST: All authors have none to declare.

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